



Original Contribution

Serum Enterolactone Concentration and the Risk of Coronary Heart Disease in a Case-Cohort Study of Finnish Male Smokers

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The lignan enterolactone produced by the intestinal microflora from dietary precursors has been hypothesized to protect against coronary heart disease. The present study examined the association between serum enterolactone concentration and the risk of coronary heart disease. A prospective case-cohort study was conducted among male smokers randomized to receive a placebo supplement in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (1986–1999). Serum enterolactone concentrations were measured by the gas chromatography-mass spectrometry method in serum collected at trial baseline from 340 men diagnosed with nonfatal myocardial infarction ($n = 205$) or coronary death ($n = 135$) during follow-up and from the randomly selected subcohort of 420 subjects. The classic risk factors-adjusted rate ratios for all coronary heart disease events in increasing quintiles of enterolactone were 1.00 (referent), 0.85 (95% confidence interval (CI): 0.51, 1.43), 0.59 (95% CI: 0.35, 1.00), 0.69 (95% CI: 0.40, 1.16), and 0.63 (95% CI: 0.33, 1.11), and the p_{trend} was 0.07. For the highest versus the lowest quintile of enterolactone, the rate ratios for nonfatal myocardial infarction and coronary death were 0.67 (95% CI: 0.37, 1.23; $p_{\text{trend}} = 0.10$) and 0.57 (95% CI: 0.26, 1.25; $p_{\text{trend}} = 0.18$), respectively. In conclusion, only weak support for the association between serum enterolactone concentration and coronary heart disease was found.

biological markers; cardiovascular diseases; cohort studies; coronary disease; diet; myocardial infarction; prospective studies; serum

Abbreviations: ATBC, Alpha-Tocopherol, Beta-Carotene Cancer Prevention; CI, confidence interval; RR, rate ratio.

The beneficial effects of a plant-based diet in the prevention of coronary heart disease are widely assumed (1). The nutrients and substances responsible for these protective effects have been extensively explored. Recently, lignans have received particular attention.

Enterolactone, the most abundant lignan in humans, is produced by intestinal microflora from dietary precursors found widely in plants (e.g., in flaxseed and other seeds, whole-grain cereals, berries, vegetables, and fruits) (2, 3). Enterolactone has been proposed to possess several biologic activities, including antioxidant activity (4), the ability to

increase hepatic low density lipoprotein cholesterol receptor activity (5), and the ability to act as an antagonist of platelet-activating factor (6), thus providing potential mechanisms for a preventive influence in coronary heart disease.

Despite much interest in lignans and enterolactone in the prevention of coronary heart disease, only a few studies have been carried out, and the results are inconsistent. A high habitual dietary intake of lignans (compared with a low intake) has been associated with a more favorable waist/hip ratio (7), coronary heart disease risk metabolic score (7), and aortic stiffness (8), whereas no association has been

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found with the concentration of total, low density, or high density lipoprotein cholesterol (7, 9) and endothelial function (10). The results concerning the intake of lignans and blood pressure (7, 10), as well as the concentration of triacylglycerols (7, 9), are inconsistent. The bulk of the evidence from nine small clinical trials suggests that supplementation of diet with flaxseed, the richest known source of lignans, can modestly reduce total cholesterol and low density lipoprotein cholesterol concentrations without a significant effect on the concentration of high density lipoprotein cholesterol or triacylglycerols (11). However, the results have not all been consistent, with the three most recent trials (12–14) showing no significant effect of flaxseed on low density lipoprotein cholesterol and only one of these trials (12) showing a significant reduction in the total cholesterol concentration. Epidemiologic evidence limits to one study where a high serum enterolactone concentration has been associated with a lower risk of an acute coronary event (odds ratio = 0.41, 95 percent confidence interval (CI): 0.22, 0.76) (15), coronary heart disease mortality (relative risk = 0.44, 95 percent CI: 0.20, 0.96) (16), and cardiovascular disease-related mortality (relative risk = 0.55, 95 percent CI: 0.29, 1.01) (16). To clarify the association between serum enterolactone concentration and the risk of coronary heart disease, we conducted a prospective case-cohort study among middle-aged men.

MATERIALS AND METHODS

Study population

The Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study was a randomized, double-blind, placebo-controlled trial the design and methods of which have been described in detail elsewhere (17). The primary aim was to determine whether daily supplementation with alpha-tocopherol, beta-carotene, or both would reduce the incidence of lung cancer and other cancers. Participants were screened by a postal questionnaire from among all men aged 50–69 years and living in the southwestern part of Finland ($n = 209,406$). A total of 29,133 male smokers (smoked at least five cigarettes per day) were randomized into one of four intervention regimens: alpha-tocopherol (50 mg/day), beta-carotene (20 mg/day), both, or neither (placebo). Recruitment began in April 1985 and continued until June 1988. The intervention lasted for 5–8 years until April 30, 1993. The participants were followed up posttrial through national registers until December 31, 1999.

The ATBC Study was approved by the institutional review boards of the National Public Health Institute of Finland and the US National Cancer Institute. Written informed consent was obtained from each study participant at baseline.

The present study was done within the placebo group of the ATBC Study ($n = 7,287$). Subjects who reported a previous myocardial infarction at baseline ($n = 438$), had missing values for risk factors of coronary heart disease (listed in table 1; $n = 724$), or either were diagnosed with acute myocardial infarction ($n = 135$) or died ($n = 86$) within 1 year after randomization were excluded, leaving 6,065 men for follow-up.

The endpoints of this study were nonfatal myocardial infarction (i.e., alive on day 28 after onset of the event) or death from coronary heart disease. Endpoints were identified through linkage with the national Hospital Discharge Register and the national Register of Causes of Death. Only the first event after randomization was registered as an endpoint. Nonfatal myocardial infarction or coronary death was diagnosed in 737 men during follow-up (mean: 11.1 years), of which one half ($n = 377$) was randomly selected for the present study.

A case-cohort study design was used to examine the association between serum enterolactone concentration and the risk of coronary heart disease. Therefore, a random subcohort of 897 men (14.8 percent) was drawn from the 6,065 eligible men, and again one half ($n = 447$) was selected for this study.

Thirty-seven cases and 27 members of the subcohort lacked a serum sample for enterolactone analysis. Thus, 340 cases (205 cases of nonfatal myocardial infarction and 135 cases of coronary death) and 420 members of the subcohort, 49 of whom were also cases of coronary heart disease, comprised the final study population.

Data collection

Baseline information on sociodemographic characteristics and medical history was collected by questionnaires. Height and weight were measured, and body mass index was computed (weight (kg)/height (m)²).

Assay of serum samples

Venous samples were taken from fasting subjects at the baseline examination. Serum was separated, divided into 1-ml aliquots, and stored in glass vials at -70°C until analysis of enterolactone. Serum enterolactone concentrations were analyzed by a method based on solid-phase extraction and detection by gas chromatography-mass spectrometry. Hydrolysis and extraction procedures were performed as previously described for serum quercetin (18). For detection of enterolactone, a new method was developed and applied. In brief, serum was incubated overnight with beta-glucuronidase-sulfatase at pH 5.5. Extraction was performed by using Bond Elut C₁₈ extraction cartridges (Varian, Inc., Palo Alto, California) as described (18). After eluting the compound with acetonitrile and drying down the eluate, we added 15 μl of a silylation reagent (*N*-methyl-*N*-trimethylsilyltrifluoroacetamide; Aldrich, Steinheim, Germany) and 85 μl of acetonitrile to the vial. Of this mixture, 1 μl was injected into the gas chromatograph-mass spectrometer, which consisted of a Hewlett Packard (Agilent, Palo Alto, California) model 6890 gas chromatograph, a Hewlett Packard model 5973 mass selective detector (electron impact at 70 eV), and a Hewlett Packard Chemstation data system. The gas chromatographic column (DB-35MS) was from J & W Scientific (Folsom, California). The gas chromatograph was operated in the splitless mode. The operation conditions were as follows: The initial temperature was 120°C for 1 minute, then raised at a rate of 15°C per minute to 320°C , and held constant for 4 minutes. Solvent delay was set at 4 minutes,

TABLE 1. Selected baseline characteristics of coronary heart disease cases and subcohort members, Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study, Finland, 1986–1999

	Cases (n = 340)		Subcohort (n = 420)	
	Mean or %	Standard deviation	Mean or %	Standard deviation
Age (years)	58.9	5.2	57.2	4.9
Body mass index (kg/m ²)	26.8	4.3	26.1	3.6
Systolic blood pressure (mmHg)	145.7	21.9	139.9	18.6
Diastolic blood pressure (mmHg)	88.4	11.3	86.8	11.3
History of diabetes (%)	7.1		4.0	
History of coronary heart disease (%)	11.8		5.0	
No. of cigarettes/day	20.3	8.6	20.0	8.4
Years of smoking	37.5	8.4	35.6	8.0
Alcohol intake as ethanol (g/week)	13.8	17.2	18.7	20.8
Serum enterolactone (nmol/liter)	17.8	19.8	18.1	17.5
Serum total cholesterol (mmol/liter)	6.5	1.1	6.3	1.2
Serum high density lipoprotein cholesterol (mmol/liter)	1.18	0.31	1.24	0.33
Total energy intake (kcal)	2,722	747	2,776	725
Whole-grain consumption (g)	215	148	201	127
Vegetable consumption (g)	94	68	106	69
Fruit (and berry) consumption (g)	106	93	111	95
Fiber intake (g)	23.7	10.2	24.1	9.5
Vitamin C intake (mg)	92.7	50.4	99.0	50.2
Folate intake (μg)	319.2	98.9	325.8	97.6
Total fat intake (g)	103.5	35.2	104.7	34.0

and helium was used as the carrier gas at a flow of 1.5 ml/minute. The injection port, transfer line, quadrupole, and ion source temperatures were set at 250, 300, 150, and 230°C, respectively. A selective ion-monitoring mode was applied. The ions used for quantitation were m/z 180 and m/z 442 for enterolactone and m/z 193 and m/z 358 for 4,4'-dihydrobenzophenone, which was used as an internal standard. Enterolactone was quantitated by comparing its peak height with that of the internal standard. Reference samples were analyzed in each run, and they were evenly divided between actual samples. The between-day coefficient of variation was 19.4 percent for a pooled reference sample (~26 nmol/liter) collected from human subjects consuming their normal diets. For another sample containing 10 nmol/liter of added enterolactone, the coefficient of variation was 16.2 percent.

Statistical methods

All analyses were performed with the statistical software R (19). The association of serum enterolactone concentration with the risk factors for coronary heart disease was examined within the subcohort only. The subcohort was divided into the quintiles of serum enterolactone (cutoff points at 5.02, 10.44, 16.43, and 28.24 nmol/liter), and the medians and proportions of the variables of interest were calculated

in each quintile. The Kruskal-Wallis test for medians and the Fisher exact test for proportions were then used to test equality across the quintiles.

The association between serum enterolactone concentration and coronary heart disease was examined by a modified proportional hazards model, with comparison of each case at the date of diagnosis with subjects in the subcohort at risk at that time, that is, risk sets consisting of one case and several referents. The rate ratios of incident coronary heart disease and 95 percent confidence intervals in relation to quintiles of serum enterolactone were computed by use of a weighted proportional hazards regression, accounting for the case-cohort design by the Barlow method (20). Whether there was a trend of increasing rate ratios across enterolactone quintiles was investigated by assessing equally spaced scores to the quintile groups and treating this as a continuous variable in the proportional hazards regression model. Both the univariate (i.e., controlling for age only) and basic multivariate (i.e., controlling for age, body mass index, total and high density lipoprotein cholesterol, diastolic and systolic blood pressure, intake of alcohol, number of smoking years and cigarettes smoked per day, history of coronary heart disease and diabetes mellitus, and fasting time) models were fitted. In further analyses, dietary factors (whole-grain cereals, vegetables, fruits and berries, fiber, vitamin C, folate, and total fat) were added to the basic multivariate model.

TABLE 2. Medians and proportions of risk factors for coronary heart disease by quintiles of serum enterolactone concentration in a subcohort (*n* = 420), Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study, Finland, 1986–1999

	Quintile 1 (0.2–5.02 nmol/liter)	Quintile 2 (5.03–10.44 nmol/liter)	Quintile 3 (10.45–16.43 nmol/liter)	Quintile 4 (16.44–28.24 nmol/liter)	Quintile 5 (28.25–106.07 nmol/liter)	<i>p</i> _{heterogeneity} [*]
Serum enterolactone (nmol/liter)	2.6	7.5	13.0	20.1	40.9	<0.0001
Age (years)	55.9	56.1	56.5	55.4	58.0	0.463
Body mass index (kg/m ²)	26.4	26.3	26.2	25.8	24.7	0.023
Systolic blood pressure (mmHg)	140	138	140	140	134	0.838
Diastolic blood pressure (mmHg)	90	87	88	88	84	0.201
Serum high density lipoprotein cholesterol (mmol/liter)	1.20	1.13	1.22	1.21	1.23	0.470
Serum total cholesterol (mmol/liter)	6.1	6.1	6.6	6.3	6.2	0.221
Alcohol consumption (g/week)	15.8	14.3	11.1	10.4	12.0	0.707
No. of cigarettes/day	20	20	20	20	20	0.126
Years of smoking	35	37	35	34	38.5	0.136
History of diabetes (%)	3.6	3.6	2.4	3.6	7.1	0.689
History of coronary heart disease (%)	4.8	3.6	6.0	4.8	6.0	0.985
Total energy intake (kcal)	3,001	2,832	2,727	2,774	2,617	0.039
Whole-grain consumption (g)	200	210	199	195	212	0.888
Vegetable consumption (g)	99	107	91	115	124	0.125
Fruit (and berry) consumption (g)	99	111	106	121	128	0.295
Fiber intake (g)	23.7	24.5	22.4	23.4	25.9	0.498
Vitamin C intake (mg)	98.3	103.5	93.5	93.6	99.9	0.801
Folate intake (μg)	348.2	330.3	311.7	317.5	322.4	0.615
Total fat intake (g)	107.6	109.8	104.8	106.7	94.8	0.056

* The Kruskal-Wallis test for medians and the Fisher exact test for proportions were used to test equality across the quintiles.

The dietary factors were energy adjusted by the residual method. Inclusion of the dietary factors did not, however, notably alter the results (data not shown), and therefore, only the results from the univariate and basic multivariate models are presented. The models were fit separately for nonfatal myocardial infarction and coronary death. All statistical tests were two sided.

RESULTS

Baseline characteristics of the cases and subcohort are shown in table 1. Compared with the members of the subcohort, incident cases of coronary heart disease were older; had higher blood pressure, body mass index, and number of smoking years; and had lower intake of alcohol. A larger proportion of cases than members of the subcohort had a history of diabetes or coronary heart disease.

The median serum enterolactone concentration in the subcohort was 13.0 nmol/liter, and there was more than a 15-fold difference in median enterolactone concentration between the highest and the lowest quintiles of enterolactone (table 2). The serum enterolactone concentration was inversely associated with body mass index (*p*_{heterogeneity} = 0.02) and intake of energy (*p*_{heterogeneity} = 0.04), while no association was found with other cardiovascular risk factors (i.e., age, blood pressure, serum total or high density lipo-

protein cholesterol, intake of alcohol, number of smoking years or cigarettes smoked per day, and history of coronary heart disease or diabetes) or dietary factors (table 2).

The mean serum enterolactone concentration among cases was comparable to that of the members of the subcohort (17.8 (standard deviation: 19.8) nmol/liter vs. 18.1 (standard deviation: 17.5) nmol/liter). After adjustment for age only, the serum enterolactone concentration was inversely associated with the risk of coronary heart disease (figure 1) (for the highest vs. the lowest quintile of enterolactone: rate ratio (RR) = 0.58, 95 percent CI: 0.36, 0.93; *p*_{trend} = 0.02). However, further adjustment for classic risk factors attenuated the rate ratios, and they were no longer statistically significant; the adjusted rate ratios from the lowest to the highest quintile of enterolactone were 1.00 (referent), 0.85 (95 percent CI: 0.51, 1.43), 0.59 (95 percent CI: 0.35, 1.00), 0.69 (95 percent CI: 0.40, 1.16), and 0.63 (95 percent CI: 0.33, 1.11), and *p*_{trend} was 0.07. No single risk factor was responsible for the attenuation. Similar results were obtained when subjects were divided into the tertiles according to their serum enterolactone concentration (for the highest vs. the lowest tertile of enterolactone: the adjusted RR = 0.78, 95 percent CI: 0.50, 1.20; *p*_{trend} = 0.22).

When subjects reporting coronary heart disease at baseline (*n* = 61) were excluded, the risk of coronary heart

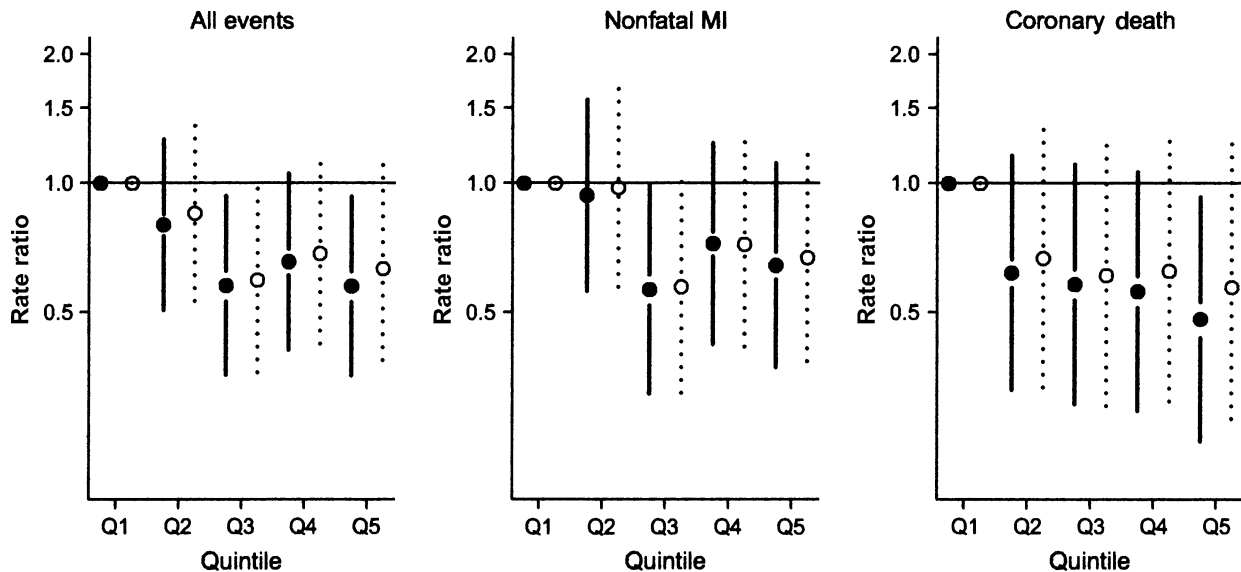


FIGURE 1. Age-adjusted (black circle) and classic risk factors-adjusted (white circle) rate ratios with 95% confidence intervals for all coronary heart disease events, nonfatal myocardial infarction (MI), and coronary death, by quintile (Q) of serum enterolactone concentration, Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study, Finland, 1986–1999.

disease was similar (for the highest vs. the lowest quintile of enterolactone: the adjusted RR = 0.75, 95 percent CI: 0.44, 1.29; $p_{\text{trend}} = 0.29$). In addition, the exclusion of subjects with diabetes mellitus ($n = 41$) at baseline did not produce substantially different results from those including the entire study population (for the highest vs. the lowest quintile of enterolactone: the adjusted RR = 0.77, 95 percent CI: 0.45, 1.33; $p_{\text{trend}} = 0.31$).

About two thirds of coronary heart disease cases were diagnosed as nonfatal myocardial infarction and one third as coronary death. No consistent association was present when data were analyzed separately according to coronary heart disease event (figure 1); the rate ratios adjusted by classic risk factors (for the highest vs. the lowest quintile of enterolactone) for nonfatal myocardial infarction and coronary death were 0.67 (95 percent CI: 0.37, 1.23; $p_{\text{trend}} = 0.10$) and 0.57 (95 percent CI: 0.26, 1.25; $p_{\text{trend}} = 0.18$), respectively.

DISCUSSION

The results of this case-cohort study support only weakly the hypothesis that a high serum enterolactone concentration is associated with the reduced risk of coronary heart disease. This finding is true for both nonfatal acute myocardial infarction and coronary death.

Our findings differ from those of the only published study about serum enterolactone and risk of acute myocardial infarction. In a prospective study of 167 middle-aged Finnish men with an acute coronary event and their matched controls, men in the highest quartile of serum enterolactone concentration (>30.1 nmol/liter) had a 59 percent (95 percent CI: 24, 78) lower risk of acute coronary event than did

men in the lowest quartile (<7.2 nmol/liter) (15). In the same study cohort, a high serum enterolactone concentration was also associated with a lower coronary heart disease-related mortality (relative risk = 0.44, 95 percent CI: 0.20, 0.96) and cardiovascular disease-related mortality (relative risk = 0.55, 95 percent CI: 0.29, 1.01) (16). We repeated our analysis by using the same cutoff points (i.e., <7.2 and >30.1 nmol/liter) as Vanharanta et al. (15) used, but this analysis, also, disclosed no significant association between the serum enterolactone concentration and the risk of coronary heart disease (data not shown).

The explanation for the different results of the present study and the previous study (15) is not clear, but may be at least partly due to the differences in study populations. First, the cases of our ATBC Study were older (mean age: 58.9 years) than were the men in the previous study (mean age: 54.2 years). The mean serum enterolactone concentrations were also slightly lower in our study population (17.8 and 18.1 nmol/liter in cases and in the members of the sub-cohort, respectively) compared with those in the previous study (18.2 and 23.5 nmol/liter in cases and in controls, respectively). This may be due to the fact that all of the subjects of the present study were smokers, who have been reported to have lower serum enterolactone concentrations than do nonsmokers (21). In the study by Vanharanta et al. (15), smoking was less prevalent (43 percent in cases and 27 percent in controls). One can also argue that smoking is such a strong risk factor itself that smokers do not benefit from enterolactone as much as do nonsmokers. Against this speaks, however, that in the previous study (15) the enterolactone-coronary heart disease association was actually stronger in smokers than in nonsmokers. Moreover, our study population included 61 men with a history of coronary heart

disease, while Vanharanta et al. (15) excluded such subjects. We reanalyzed our data by excluding subjects with a history of coronary heart disease, but the results did not differ from those of the whole study population. The analytical methods were also different. We used a gas chromatography-mass spectrometry method, which is a method of high selectivity and specificity, while Vanharanta et al. (15) used a commercial kit based on a time-resolved fluoroimmunoassay. A rather good correlation between these methods has been reported previously (22), and therefore it is unlikely that the use of different analytical methods would have caused the differences in outcomes of these two studies.

Regarding the main risk factors of coronary heart disease, only body mass index was associated with serum enterolactone concentration. The median of body mass index was highest in the lowest quintile of enterolactone and was linearly decreased in increasing quintiles of enterolactone. We also found that intake of energy was inversely associated with the serum enterolactone concentration. Thus, healthy dietary habits (i.e., low intake of energy and high intakes of vegetables and other healthy foods with a high content of lignans) at least partly explain the association between serum enterolactone concentration and body mass index. Moreover, enterolactone has been shown to pass through the preadipocyte's cell membrane (23, 24), which means that, in subjects with a high body mass index, enterolactone may be "diluted" because of rapid transport into the cells, resulting in lower serum concentrations. In contrast to the study by Vanharanta et al. (15), this study found no association between the serum enterolactone concentration and blood pressure.

An important strength of our study is its prospective case-cohort design. The use of a random sample of participants from the ATBC Study cohort as a comparison group, instead of specifically chosen healthy controls, gives the study a clear inferential base. The generalizability of the results is, however, somewhat restricted, because the study included only smokers who participated in a clinical trial. The prospective design of the study ensured that the blood samples used were obtained up to 13 years prior to the diagnosis of coronary heart disease and ensured identical collection and handling of blood samples from cases and subcohort members. Moreover, the number of cases was relatively high, and endpoints were identified through linkage with the national Hospital Discharge Register and the national Register of Causes of Death that are shown to be highly predictive of a true major coronary event defined by strict criteria (25).

Our study has some limitations. A potential limitation is the lack of information on antibiotic use by study participants. It has been observed previously that the use of antibiotics reduces the serum enterolactone concentration (26, 27). On the other hand, it has been hypothesized that antibiotics may protect against coronary heart disease (28). Another potential weakness is that only one enterolactone measurement was available. Some studies have found a moderately high reliability coefficient for a single measurement of enterolactone of 0.84 for 2 days (29) and of 0.55 for 2 years (30), but opposite observations have also been reported, concluding that a single measurement of entero-

lactone is inadequate (31, 32). Our serum samples were collected after an overnight fast, which reduces day-to-day variation (31).

In conclusion, we found only weak support for the association between serum enterolactone concentration and the risk of coronary heart disease.

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Conflict of interest: none declared.

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